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# Analysis of Double-stranded Polymerase Chain Reaction Products from the *Bacillus cereus* Group by Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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The analysis of polymerase chain reaction (PCR) products by electrospray ionization-Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR) has been achieved. Specifically, a 105 base-pair nucleotide portion of the ribosomal spacer region was amplified in two members of the *B. cereus* group (i.e. *B. thuringiensis* and *B. cereus*) using PCR. These amplified regions were then analyzed by gel electrophoresis and ESI-FTICR. Based on the predicted sequence of the PCR products for each organism, the mass measurement using ESI-FTICR matched the theoretical mass within experimental error and was consistent with gel electrophoresis results. In contrast, for the typical several hour time-scale of the gel electrophoresis experiment, the mass spectrometric analysis was completed in a matter of minutes. To our knowledge, this constitutes the first report demonstrating the ionization and detection of a double-stranded PCR product by ESI-MS. This preliminary result indicates the potential use of ESI-MS to analyze PCR products on a rapid time-scale, with potential for medical and taxonomic applications.

Molecular taxonomic investigations utilize either size or sequence variations of shared genomic regions as a basis for comparison of different species.<sup>1,2</sup> The *B. cereus* group (i.e. *B. anthracis*, *B. cereus* and *B. thuringiensis*) contain organisms with both medical and agricultural relevance. *B. anthracis* and *B. cereus* are human pathogens,<sup>3,4</sup> whereas *B. thuringiensis* has been utilized as a biological pesticide.<sup>5</sup> The *B. cereus* group are highly conserved both phenotypically and genetically; therefore, differentiation of the members of this group has proven to be difficult using molecular methods.<sup>6-8</sup> The 16S/23S ribosomal RNA spacer region has shown sufficient variability to differentiate species in several other genera of prokaryotes.<sup>1,9,10</sup> The ribosomal spacer region in the *Bacilli* has displayed some variability based on available sequence information.<sup>11,12</sup> Therefore, polymerase chain reaction (PCR) amplifications have been employed to generate double-stranded products from this region, which are unique to the *B. cereus* group. Any differences that exist within this region between the species of this group cannot be resolved by gel electrophoresis.<sup>8,10,11</sup> Furthermore, the analysis of PCR products by electrophoresis is a time-consuming process requiring several hours. Therefore, we have investigated mass spectrometry as a technique which could allow for the detection of these products rapidly.

The ability to ionize and measure the mass-to-charge ratios of large biopolymers has become feasible with the advent of electrospray ionization (ESI)<sup>13</sup> and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.<sup>14</sup> Single-stranded nucleic acids of up to several hundred nucleotides in length have been successfully analyzed using MALDI ionization.<sup>15,16</sup> Furthermore, enzymatically

digested nucleic acids have been analyzed using MALDI, demonstrating that nucleic acids of this size could be analyzed after removal from reaction mixtures.<sup>17,18</sup> These studies have been extended to include PCR products amplified from human DNA.<sup>19-22</sup> Although there have been several reports of gas-phase ions produced from PCR products using MALDI, there is an absence of similar reports utilizing ESI. One reason for this is that metal-ion adduction, even for carefully 'desalted' samples, can substantially degrade the ESI mass spectra for larger nucleic acids.<sup>23</sup> Secondly, MALDI spectra are inherently simpler to interpret than ESI spectra when multiple components are present. The larger ions from the PCR product appear in a higher *m/z* range than the reaction components, that are smaller in mass.<sup>22</sup> However, the MALDI process produces ions of single-stranded DNA with decreasing ionization efficiency as mass increases.<sup>17,18</sup> Several reports exist using MALDI for the analysis of double stranded DNA and PCR products, albeit with detection of the single strands.<sup>24-27</sup> However, the analysis of an intact double-stranded DNA molecule by MALDI was recently reported.<sup>28</sup>

The gentle nature of ESI in conjunction with mass spectrometry has allowed the analysis of intact proteins, peptides, oligonucleotides and has, more recently, been used to explore protein conformation.<sup>29,30</sup> The utility of ESI-MS for the analysis of noncovalent associations of biopolymers, including single- and double-stranded DNA, as well as protein-DNA complexes has been demonstrated.<sup>31,32</sup> Although the relationship of the observed complexes to those in physiological solutions has endured much scrutiny, rapidly growing information supports the conclusion that the observed complexes originated in solution. Mass spectrometry has been employed for many types of oligonucleotide analyses including DNA adduction levels

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for cancer screening,<sup>33</sup> sequence verification<sup>34,35</sup> sample purity,<sup>36</sup> and radiation-damage product identification.<sup>37,38</sup> Single-stranded DNA molecules have been detected with 50 to 100 residues using both ESI<sup>32</sup> and MALDI,<sup>39</sup> and, in fact, species as large as 100-mers have been investigated using ESI-FTICR for sequence verification.<sup>39</sup> However, due to lower ionization of double-stranded DNA molecules, ESI mass spectrometry has only been successful for the detection of species with as many as 20 base pairs (bp).<sup>40,41</sup> Interestingly, the analysis of DNA with mass spectrometry seems to be dominated by the analysis of synthetic oligonucleotides, in spite of current efforts involved in the sequencing of the human genome using mass spectrometry<sup>42</sup> and the obvious benefits of PCR.

In general, PCR reactions utilize oligonucleotide primers which are complementary to sequences that flank a targeted area on a double-stranded template.<sup>43</sup> The region flanked by these primers is copied millions of times during the course of repetitive reaction steps.<sup>43</sup> The specific production of double-stranded fragments from conserved genomic regions has allowed this technique to be used as a diagnostic tool.<sup>44,45</sup> In the current report, a taxonomically significant PCR product from the *Bacilli* has been used. This product was designed to be of a size amenable to mass spectrometry. This PCR product has been successfully analyzed by FTICR-MS from two different species of *Bacilli*. Additionally, the feasibility of obtaining an accurate mass measurement on double-stranded PCR products of this size has been examined, for the purpose of distinguishing slight differences in mass. To our knowledge, this is the first successful mass spectrometric analysis of a PCR product using ESI. Furthermore, the results clearly indicate that large (>100 bp) dsDNA can be ionized and detected by ESI-FTICR mass spectrometry.

## EXPERIMENTAL

### Materials

*B. thuringiensis* strain 4C2 was obtained from the *Bacillus* Genetic Stock center (Ohio State University, Columbus, OH, USA), and *B. cereus* strain 6464 was obtained through the American Type Culture Collection (ATCC). These strains were previously characterized using a variety of taxonomic methods. Among these were several morphological and physiological comparisons, whole cell carbohydrate profiling using gas chromatography/mass spectrometry, and gel electrophoresis comparisons of PCR products derived from the entire ribosomal spacer region.<sup>4</sup>

Primers corresponding to positions 1484 to 1498 from the 3' end of the 16S rRNA and positions 48 to 62 of the *B. cereus* 16S/23S rRNA spacer region were synthesized with the sequences 5'-caaggtagccgtatc-3' and 5'-aaaactgaa-aaaaac-3', respectively.<sup>46</sup> The sequence for the 16S rRNA primer was derived from the *B. subtilis* 16S sequence<sup>47</sup> while the 16/23S spacer region primer was derived from *B. cereus* ATCC strain 14579.<sup>12</sup> These primers as well as the mixed-base synthetic 49-mer were synthesized at the University of South Carolina oligonucleotide synthesis facility. The mass of the 49-mer based on its sequence was calculated to be 14 781.56 Da.

The materials required to perform the PCR amplifications were purchased from Amersco, Inc. (Solon, OH, USA). These materials were obtained in a kit containing MgCl<sub>2</sub>, dNTPs (dATP, dGTP, dCTP, and dTTP), 2 units/μL

thermostable DNA Polymerase, and 10X PCR buffer (500 mM KCl, 0.1% triton X-100, and tris-HCl at pH 8.6).

The 3:1 agarose was purchased from Amresc . Inc. The 50X Tris Acetate ethylenediamine-tetraacetic acid (EDTA) buffer (TAE), consisted of 1 M tris-HCl at pH 8.6, 40 mM acetic acid, and 1 mM EDTA. The pGEM<sub>T</sub> double-stranded DNA markers for electrophoresis were purchased from Promega Corp. (Madison, WI, USA). The gel loading consisted of 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol.

The diaminocyclohexane-tetraacetic acid (CDTA), piperidine and imidazole were purchased from the Sigma Chemical Co. (St. Louis, MO, USA), while the triethylamine (TEA) was purchased from Fisher Scientific (Fairlawn, NJ, USA) in the highest purity available. Microcon-10 membranes possessing a 10 000 MW cutoff were purchased from Amicon Corp. (Beverly, MA, USA).

### Methods

The chromosomal DNA was prepared from each of the bacterial strains after growing the organisms in 500 mL nutrient broth cultures for 16 h. The method for DNA preparation has been previously described.<sup>4</sup> Briefly, this involves breaking the cells open using successive rounds of boiling and freezing. The cell lysates were incubated first with an RNase, followed with a protease. Phenol/chloroform extractions were used to remove any hydrophobic cellular components, and then the remaining nucleic acids were precipitated using 95% ethanol.

The PCR amplifications were carried out in 100 μL volumes in a Barnstead/Thermolyne thermocycler (Thermolyne/Barnstead Dubuque, IA, USA). The reactions contained approximately 1 μg of chromosomal DNA preparation with 100 pM of each primer, 1 mM dNTPs, 5 mM MgCl<sub>2</sub>, and PCR buffer containing 50 mM KCl, 0.01% Triton X-100, and tris-HCl at pH 8.6. The program used for amplification consisted of a 1 min denaturation step at 95 °C, 3 min annealing step at 50 °C, 2 min ramp to 72 °C and a 2 min extension step at 72 °C.

The PCR products from each species were concentrated by placing 10 reaction volumes (1 mL total volume) on three separate Microcron-10 membranes. TEA was added to a concentration of 1% and CDTA was added to a final concentration of 5 mM to each membrane. Nanopure water was used to bring the volume up to 500 μL. These filters were centrifuged for 30 min at 10 000 g. This procedure was repeated three additional times followed by a fifth spin using 0.01% TEA and 5 mM CDTA in the 500 μL volume. The PCR products were collected by inverting the membranes and centrifuging at 4000 g for 10 min. A volume of ~ 15 μL was collected from the membranes.

Gel electrophoresis was carried out using a 3%-by-weight agarose gel for 2.5 h at 60 V. 1 μL of the collected PCR concentrate was added to 2 μL of the loading buffer and loaded onto the gel. The two outer lanes of the agarose gel were loaded with 2 μL of the pGEM double stranded DNA markers.

The spectrophotometric measurements were carried out on a Beckman (Fullerton, CA, USA) 640 spectrophotometer. Absorbances was measured at 260, 280 and 320 nm. A blank PCR mixture which has been prepared similarly was used as the calibrant for the absorbance measurements. The amount of PCR product present was estimated based on the absorbance at 260 nm following the cleaning and concentration of the products.

## Mass spectrometry

Two mass spectrometers were used to complete these studies. The first was a VG Quattro (Beverly, MA, USA) triple quadrupole mass spectrometer. A source potential of 2.3 kV was applied to produce a stable negative ESI current. The capillary temperature was held constant at 100 °C for all experiments unless otherwise noted. A 50 µL/min flow of 50/50 acetonitrile + water was used for sample delivery. Injection volumes of 20 µL were used to acquire each spectrum and data were acquired for 3 min over a 1200  $m/z$  range at 5 s per scan.

The ESI-Fourier transform ion cyclotron resonance mass spectrometer used for the present study has been described in detail elsewhere.<sup>49</sup> The methods employed for the detection of the weak signals produced by the PCR products, including filtered-noise waveforms and quadrupole excitation (QE) for enhanced ion accumulation performance, have also been previously reported.<sup>49,50</sup> All waveforms used for broadband QE accumulation windows were typically employed at 5 to 10 volts peak-to-peak (Vp-p). For selected-ion accumulation, single frequency QE events were employed with amplitudes of 0.5 Vp-p.

The negative-ionization-mode ESI was performed in the presence of a co-axial SF<sub>6</sub> sheath gas to suppress discharge. The product of 10 PCR reactions was combined to make a total volume of ca. 20 µL. Aliquots of this solution were then diluted to twice the original volume with 10 mM NH<sub>4</sub>Ac prior to analysis. The samples were infused at 0.3 µL/min and a stable spray was maintained at -2 to -2.5 kV.

## RESULTS AND DISCUSSION

The sequence from the 3' end of the 16S rRNA gene from *B. cereus* and *B. thuringiensis* has been described up to nucleotide position 1486.<sup>51</sup> Additional reports have extended the sequence of the 16S rRNA gene to its end for a strain of both *B. thuringiensis* and *B. cereus*.<sup>43</sup> The sequence of the 16/23S spacer regions for several strains of each species have also been described in their entirety.<sup>11,12</sup> Based on this sequence, the predicted size of the resultant double-stranded PCR product from *B. cereus* is 105 base pairs and the predicted molecular weight is 64 753.2 Da. A number of the strains of *B. thuringiensis* have been reported to lack the thymine at position 32 similar to the reported sequences for *B. cereus* strains.<sup>11</sup> A double-stranded PCR product which contains the additional base pair would have a molecular mass of 65 370.7 Da. The remainder of the spacer region sequence has been reported to be identical between the two species. However, the sequences for only two strains of *B. cereus* have been obtained for this region. To estimate the number of bases in the PCR products, the amplified regions were analyzed on a 3% agarose gel using the marker kit. The migration of dsDNA marker kit was fitted using a 2nd-order polynomial expansion and resulted in a good correlation ( $r_0 = 0.996, N = 8$ ). Using this standard curve, the *B. thuringiensis* and *B. cereus* products were determined to be 101 base pairs in length with an estimated %RSD of 5%. This indicates the length is consistent with the predicted sequence; however, the gel data cannot provide accurate mass or sequence information.

The analysis of the PCR products by ESI mass spectrometry requires substantial clean up to reduce the concentrations of other components in the reaction mixture, some of which are present in molar excess to the double-stranded product. In order to prevent severe ion-signal

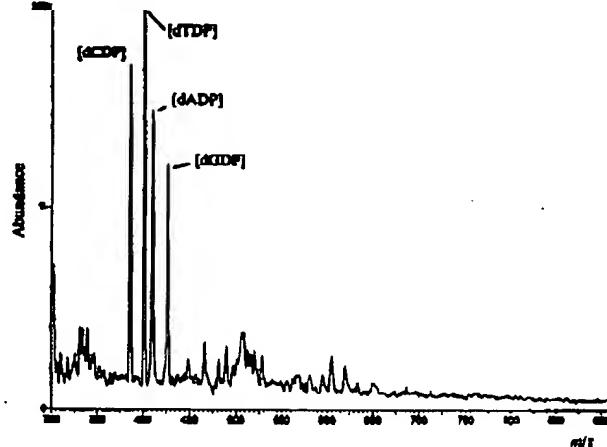
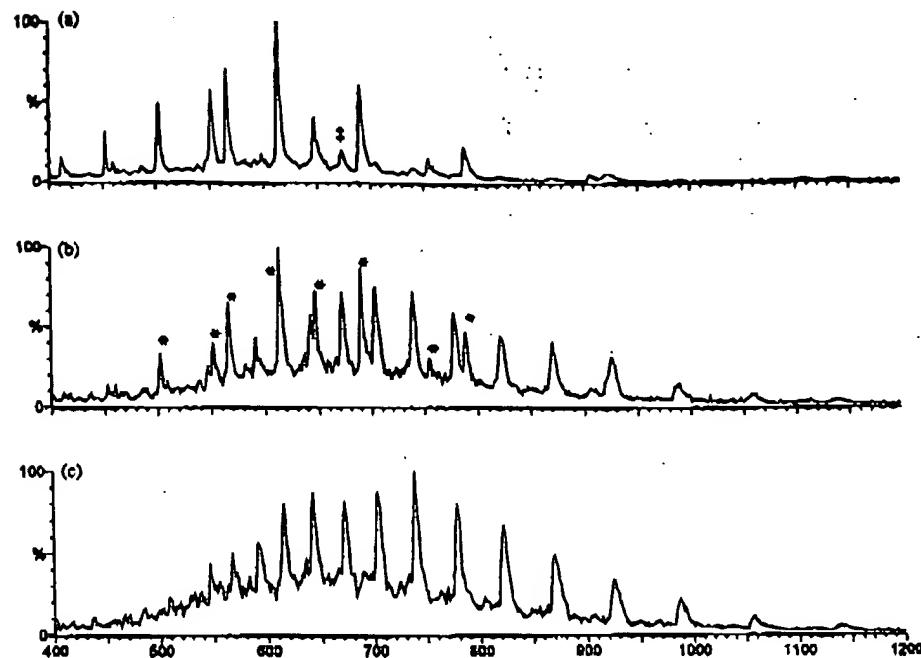


Figure 1. ESI quadrupole mass spectrum produced from the direct injection of a PCR reaction mixture containing 135 pM of a synthetic mixed base 49-mer without any prior cleanup steps. The predominant masses in this spectrum correspond to the four dNTPs. Notice that neither the 49-mer nor the smaller oligonucleotide primers produced any peaks in the presence of the large amount of dNTPs.

suppression of this product, the concentration of the much smaller dNTPs and oligonucleotide primers in particular, must be drastically reduced. To illustrate this, 2 µg of a mixed base 49-mer (135 pM) was placed with a PCR reaction mixture and analyzed by ESI-MS. Figure 1 shows the mass spectrum obtained from this sample with the dominant peaks originating from the four dNTPs. Neither the masses originating from the 49-mer nor those of the 15- and 18-mer oligonucleotide primers (4520.12 and 5529.83 Da, respectively) were evident in the spectrum. Similarly, the effect of the primers on the ionization of the 49-mer standard was examined and the result is shown in Fig. 2. The presence of various amounts of the primers relative to the 49-mer illustrates a need for either the ability to analyze trace species or the need to have a vast excess of the higher-molecular-weight nucleic acids relative to the primers for analysis by ESI-MS. Despite being present at less than one third of the concentration of the 49-mer in Fig. 2(b), the oligonucleotide primers still produce peaks of greater intensity than those arising from the 49-mer. This is likely to be increasingly important for molecules of greater size such as a PCR product of over 100 base pairs in size. It is interesting to note, however, that for FTICR the relative intensities of the oligonucleotides will be larger by a factor equivalent to the number of charges; thus, detection of the larger oligonucleotides will be favored. Additionally, the yield of the PCR reactions were on the order of 17 to 39 pmol of material for a 105 base pair nucleic acid. This estimate is based on the average amount of material collected for thirteen reactions, using the absorbance observed at 260 nm.

The analysis of the *B. thuringiensis* PCR product by ESI-FTICR resulted in the spectrum shown in Fig. 3. These data were acquired with a 20 s accumulation using QE with broadband noise excitation corresponding to the  $m/z$  range 1400–2000. Other ions were observed from this sample, such as the 4-charge state ions at  $m/z$  1145 corresponding to one of the primers used in the reaction, when waveforms covering broader  $m/z$  ranges were used for accumulation. In Fig. 3, the charge states are resolved and are assigned to the intact PCR product; however, due to the small ion populations, the high resolution FTICR typically affords was not achieved in this initial study. Calculation of the mass using the charge-state distribution resulted in a

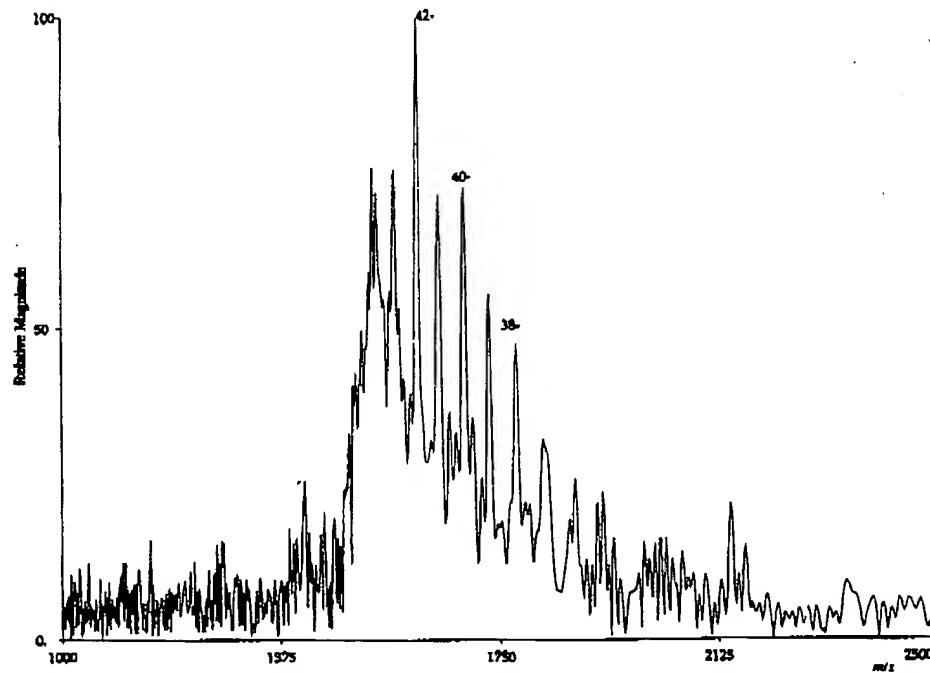
## ANALYSIS OF POLYMERASE CHAIN REACTION PRODUCTS BY ESI-PTICRMS



**Figure 2.** Comparison of various mixtures of the synthetic 49-mer and the oligonucleotide primers 5'-csaggccatccacgt-3' (4520.12 Da) and 5'-gtttttttggaaaacttgt-3' (5529.83 Da). (a) 100 pM of each primer with 36 pM of the synthetic 49-mer. All peaks are assignable to the primers with the exception of the peak labeled '‡' which is assignable to the 49-mer. (b) 100 pM of each primer with 360 pM of the 49-mer. Primers are indicated with asterisks (\*) and the unlabeled peaks are assignable to the 49-mer. (c) 10 pM of each primer and 360 pM of the 49-mer. All peaks are assignable to the 49-mer. All samples have TEA added to a concentration of 0.01% prior to infusion into the ESI source.

molecular mass of  $67\ 586 \pm 1703$  Da, the error being reported as the confidence interval of the mean at 99% confidence limits. The large uncertainty associated with the mass measurement arises from the difficulty in unambiguously assigning charge states ( $\pm 1$  charge) which is attributed to the broad peaks that clearly reflects the contributions due to heterogeneity and/or adduction of

cations to the phosphate backbone as well as the short transient. A large amount of potassium adduction is likely since 50 mM potassium chloride was present in the reaction mixture. The mass calculated from the two possible sequences for the *B. thuringiensis* PCR product are 64 753.23 and 65 370.66 Da, which indicated that the mass measurement is correct within experimental error. The mass

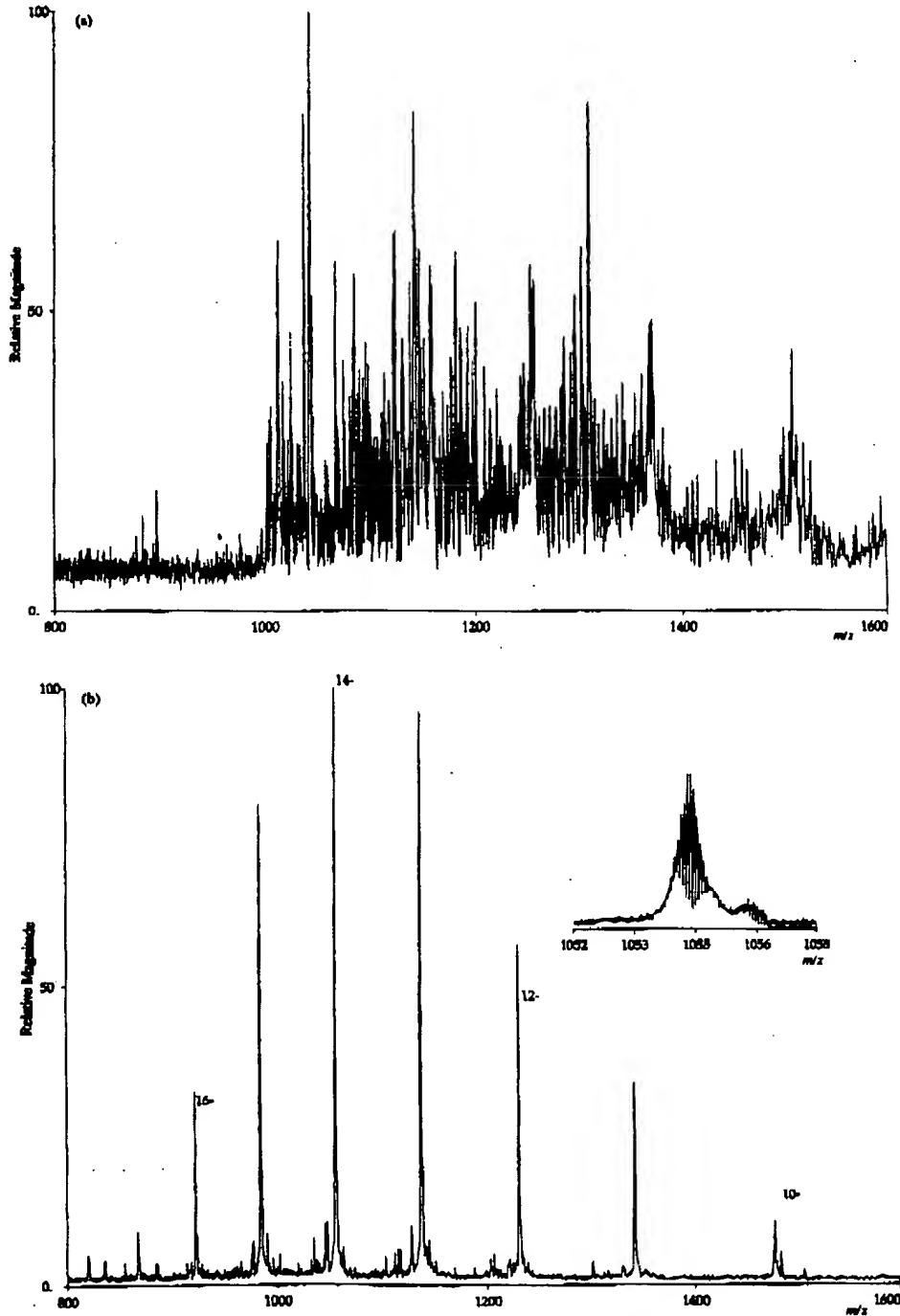


**Figure 3.** The ESI-PTICR mass spectrum of the double stranded 105 base pair polymerase chain reaction product of the 16S/23S RNA spacer region of the bacterial *B. thuringiensis* with tentative charge states indicated. This spectrum was acquired with a 20 s selective accumulation using quadrupolar noise excitation corresponding to the  $m/z$  range 1400–2000 and produces a mass measurement of  $67\ 586 \pm 1703$  Da.

differences between these two values and the calculated value were 2833 and 2215 Da, respectively. Although the sequence of the products should be comparable to a different *B. cereus* strain which is known, variations in base composition and length may also account for some level of the measured error. Given these multiple sources of error and uncertainty, the FTICR mass measurements are within the estimated precision. It is interesting to note that if the charge states are all decreased by one (the estimated precision), the calculated molecular mass is determined to be 65 942 Da which would then be in close agreement to the expected values listed above.

Similarly, the PCR product from *B. cereus* was also analyzed by both gel electrophoresis and ESI-FTICR. The length was determined to be 101 base pairs by gel electrophoresis indicating that it is similar in size to the *B. thuringiensis* product within the error of the measurement. The mass determined based on its predicted sequence is 64 753.2 Da and that calculated from the FTICR spectrum was  $65\ 401 \pm 1683$  Da, a difference of 648 Da, which is within the experimental error due to the complications noted above.

The ESI-FTICR mass spectral data should be improved by co-addition of piperidine and imidazole to the solution to



**Figure 4.** (a) The ESI-FTICR spectra of mixed-base 49-mer infused from a solution containing 80 vol % acetonitrile acquired with a 10 s selective-accumulation with quadrupolar noise excitation corresponding to the  $m/z$  range 1000–2000. (b) ESI-FTICR spectrum produced with a 3 s accumulation and a similar solution as in Fig. 2(a) except for the addition of 25 mM piperidine and 25 mM imidazole. The inset in the spectrum shows an expansion of the 14- charge state.

be infused into the spectrometer.<sup>52</sup> It has previously been shown that the extent of cation adduction on small oligomers (< 20-mer) can be substantially decreased by adding these two organic bases to the solution.<sup>52, 53</sup> However, a recent study showed that extensive adduction of piperidine to the oligonucleotide could be observed in the ESI mass spectrum at reduced capillary temperature.<sup>53</sup> It was uncertain whether extensive adduction would be problematical for larger oligomers (i.e. under normal interface conditions and capillary temperatures, if piperidine could be removed from the oligonucleotide). To investigate the possibility of using piperidine and imidazole to improve the quality of larger DNA oligomers, a mixed base 49-mer was analyzed with and without the use of the organic bases. Figure 4(a) shows the spectrum from a 10 s QE accumulation over the  $m/z$  range from 1000 to 2000. For this spectrum, the 49-mer was dissolved in an aqueous solution containing 80% acetonitrile. Many peaks are evident here, with perhaps a slight indication of charge states near the region  $m/z$  1200–1400. However, the degrading effect of multiple adduction on the resultant mass spectrum is clearly evident with the comparison to the mass spectrum in Fig. 4(b). This spectrum was obtained from a solution contained the same amount of oligonucleotide as the spectrum in Fig. 4(a); however, 25 mM piperidine and 25 mM imidazole were added to reduce adduct level formation and enhance signal intensity, respectively. Better resolved charge states are evident, as are the isotopic contributions in each charge state, leading to a molecular weight measurement of 14 781.1 Da for the most abundant isotopic peak. Furthermore, even though the signal-to-noise ratio in Fig. 4(b) is much greater than that in Fig. 4(a), only a 3 s accumulation covering the mass range 800–1600 was required for this spectrum. This observation highlights the general problems encountered with larger biomolecules and adduction, and the resultant poor signal-to-noise ratios. That is, even though a large number of ions may be formed, the dispersal of the ion current between species of many different masses, charges, and extent of adduction results in poor signal intensities. The addition of piperidine and imidazole resulted in effective adduct removal for the 49-mer in this study, as well for many smaller oligonucleotides<sup>49, 50</sup> with no observation of piperidine adduction to the larger oligomer. The addition of piperidine and imidazole was not used for the analysis of the PCR products not only because it is larger but also because it is unclear if the dsDNA will chemically 'melt' in solution using this buffer system. However, the effect of piperidine and imidazole on the ionization of PCR products is currently under investigation.

The present results clearly indicate that ESI-FTICR mass spectrometry can be employed to investigate ds-DNA produced from a PCR reaction. This study demonstrates that the method can provide rapid information on PCR products that have been characterized by gel electrophoresis. The analysis of these products by ESI-FTICR required only a matter of seconds whereas the information from the electrophoresis required several hours. Using mass spectrometry for the analysis of PCR products is not only rapid but also lends itself to automation which may be useful for medical applications. The precision of the mass measurement of the *B. thuringiensis* by ESI-FTICR was estimated to be 1703 mass units (as a result of the uncertainty in assigning charge states), or 3–4 base pairs in size, which is roughly equivalent to the precision of the gel. Before the apparently slight differences in the spacer region can be

differentiated, better mass precision is required. Improvements by the reduction or elimination of metal cation adduction using piperidine and imidazole may prove useful in reaching this goal. Alternatively, cation exchange columns may be useful to efficiently remove salts present in the PCR reaction mixture. Although further progress is needed to improve the precision of the mass measurements, this is a significant step towards the analysis of PCR by electrospray ionization mass spectrometry. Not only can these significantly large ds-DNA molecules be observed intact in the gas phase (105–106 base pairs), but (to our knowledge) this is the first application of ESI-MS to PCR products.

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